CHEMIJA, 2004. T. 15, Nr. 1, 22–27

Synthesis and study of chitosan and poly(ethylene glycol) graft copolymers containing triazine moiety

Natalija Gorochovceva[1], Rûta Kulbokaitë[1], Remigijus Juðkënas[2] and Rièardas Makuška[1]

[1] *Department of Polymer Chemistry, Vilnius University, Naugarduko 24, LT-2006 Vilnius, Lithuania*
E-mail: natalija.gorochovceva@chf.vu.lt
[2] *Institute of Chemistry, A. Goštauto 9, LT-2600, Lithuania*

Novel chitosan-*O*-poly(ethylene glycol) graft copolymers with different graft density were synthesized. Trichlorotriazine was used as a coupling reagent for a covalent attachment of poly(ethylene glycol) monomethyl ether (MPEG) to chitosan. The chitosan etherification reaction was carried out in dimethylformamide in the presence of silver oxide. Chitosan-*O*-poly(ethylene glycol) graft copolymers were characterized by FTIR and NMR spectroscopy, X-ray diffraction and elemental analysis. Chitosan derivatives were soluble in water and aqueous solutions of a wide pH range. The reduced viscosity of aqueous solutions of the graft copolymers was extremely low and similar to that of MPEG.

Key words: chitosan, trichlorotriazine, O-PEGylated chitosan, water-soluble, viscosity

INTRODUCTION

Chitosan is a polysaccharide widely distributed in nature. It could be regarded as an analogue of cellulose with the amino group instead of the C(2) hydroxyl group, but despite its structural similarity to cellulose, it appears to be much less accessible to potential reactants than cellulose does. Thus derivatization of this polymer normally requires much more drastic conditions than are necessary for cellulose [1].

Chemical modifications of chitosan are widely used to obtain new derivatives with unique properties. There are a lot of publications concerning chitosan modifications through NH_2 groups. However, chemical modifications of this type change the fundamental skeleton of chitosan, and the modified chitosan loses its original physicochemical and biochemical activities [2]. An alternative way of chitosan derivation is its alkylation through OH groups, which is used much less.

Poly(ethylene glycol) (PEG) is regarded as one of the most suitable graft-forming polymers because of its unique properties including solubility in both water and organic solvents, low toxicity, good biocompatibility and biodegradability [3]. PEG was grafted onto chitosan by different techniques resulting in *N*-substituted chitosan derivatives [4–7]. No publications were found on PEGylation of chitosan through its –OH groups. Before grafting, PEG must be "activated", *i.e.* its terminal OH groups have to be converted to more reactive halogen, aldehyde and similar groups. Reactive intermediates possessing reactive groups (*e.g.*, 2,4,6-trichloro-1,3,5-triazine) are coupled to PEG in some cases.

2,4,6-trichloro-1,3,5-triazine (cyanuric chloride) is a heterocyclic organic compound commonly used for immobilization of proteins on various polymeric supports, such as cellulose [8] and agarose, as well as for modification of proteins [9], liposome [10] and natural polymers (*e.g.*, chitosan [11] and wool [12]). Trichlorotriazine derivatives have found extensive use in the synthesis of "activated" dyes, whiteners, herbicides, pharmaceuticals, etc. [13]. Dichlorotriazine is not as reactive as the initial trichlorotriazine, but has a sufficient residual activity for attaching to biopolymers in weakly alkaline aqueous solutions at 35–40 °C. Both mono- and dichlorotriazines exhibit biological activity and some carcinogenic properties [14]. Trichlorotriazine and its derivatives easily react with amines and alcohols (aromatic and aliphatic) in organic, organic/aqueous and aqueous solutions. Because of partial hydrolysis of trichlorotriazine and its derivatives in aqueous solutions, the use of organic media is preferable.

In this paper, we report on the covalent attachment of poly(ethylene glycol) monomethyl ether to chitosan using trichlorotriazine as a coupling reagent. The novel chitosan-*O*-MPEG graft copolymers are expected to combine the beneficial properties of chitosan and PEG and may find applications in several fields including biotechnology, pharmaceuticals, water treatments, etc.

EXPERIMENTAL

Materials

Chitosan (M_r 400000, degree of deacetylation (DD) 72%) and poly(ethylene glycol) monomethyl ether (MPEG) (M_r 2000) were obtained from FLUKA. Trichlorotriazine was obtained from Aldrich. Ag_2O was synthesized from $AgNO_3$ and KOH in our laboratory just before use. Dimethylformamide (DMF) was "dried" for two days with KOH, distilled from CaH_2 under reduced pressure and stored over 4 Å molecular sieves.

Preparation of N-phthaloyl chitosan [2]

A mixture of chitosan (5.00 g, 31.0 mmol) and phthalic anhydride (PHA) (13.8 g, 93.1 mmol) in DMF (100 ml) was heated with stirring at 130 °C under nitrogen atmosphere. After 5–7 h the mixture became a clear and viscous solution. The precipitate obtained by pouring the solution into ice-water was collected by filtration, extracted with ethanol in Soxhlet's apparatus, and dried in air to give 7.6 g of N-phthaloyl chitosan.

Preparation of 2-O-MPEG-4,6-dichloro-s-triazine (MPEGT) [15]

2,4,6-trichloro-*s*-triazine (5.5 g, 0.03 mol) was dissolved in anhydrous benzene containing 10 g of anhydrous sodium carbonate. MPEG (19 g, 0.01 mol) was added and the mixture was stirred overnight at room temperature. The solution was filtered, and diethyl ether was poured slowly into the filtrate under stirring. The obtained product was filtered off, reprecipitated several times from benzene to diethyl ether and dried in a vacuum oven.

Preparation of O-substituted chitosan – MPEGT graft copolymers

A mixture of N-phthaloyl chitosan (0.9 g, 3.35 mmol), MPEGT (14.03 g, 6.7 mmol) and Ag_2O (1.55 g, 6.7 mmol) in 60 ml of DMF was heated at 60 °C under magnetic stirring for 16 h. Then hydrazine monohydrate (40 ml) and water (80 ml) were added to the reaction vessel and the mixture was heated under magnetic stirring for 15 h at 90 °C. To remove hydrazine monohydrate the mixture was evaporated using a rotary evaporator until viscous solution. The residual solution was diluted with water and evaporated again. This procedure was repeated three times. Then the residue was diluted with water and dialyzed against water for 96 h using visking dialysis tubing (SERVA). The dialysed solution was concentrated until a solid residue using a rotating evaporator and the obtained product was dried in a vacuum oven to give 8.2 g of *O*-PEGylated chitosan.

Determination of the degree of O-substitution of chitosan

The degree of *O*-substitution of MPEG to a monosaccharide residue of chitosan (DS, %) was calculated by two independent methods, *i.e.* according to the content of primary amino groups and the content of PEG units in the copolymers determined experimentally.

The content of primary amino groups was determined by potentiometric titration [16]. A copolymer (0.1–0.2 g) was accurately weighed, dissolved in 20 ml of aqueous 0.1 N HCl and the solution was titrated with 0.1 N NaOH. A blank experiment was carried out in the same conditions. The content of amino groups (NH_2, %) in a copolymer was calculated as follows:

$$NH_2 = \frac{(V_1 - V_2) \times C \times E}{m \times 1000} \times 100,$$

where: V_1 and V_2 – the volume (ml) of NaOH solution used for titration of blank and sample solutions respectively;

C – the concentration (mol/l) of NaOH solution;

E – the molecular weight of the amino group (16);

m – the sample weight, g.

The content of PEG units in a copolymer was determined by a colorimetric method based on the partitioning of a chromophore present in ammonium ferrothiocyanate reagent from the aqueous to the chloroform phase in the presence of PEG [17]. Five millilitres of chloroform, 5 ml of ammonium ferrothiocyanate reagent and 0.5 ml of a copolymer (0.02 g/25 ml of water) solution were mixed vigorously for 30 min at room temperature. The phases were separated, and the absorbance of the lower chloroform phase was recorded at 510 nm using a cuvette with 1 cm optical path length. The method gave a linear response over a range of 0.05–0.4 mmol of MPEG-2000.

The content of MPEGT grafts containing triazine moiety (MPEGT, %) in a copolymer was calculated as follows:

$$MPEGT = \frac{c \times 2094}{m} \times 100,$$

where: c – concentration of MPEG-2000 in the sample solution determined from the calibration curve, mol/l;

2094 – molecular weight of a MPEGT graft containing triazine moiety;

m – the sample weight, g.

DS (%) was calculated referring to both above methods by the following equations:

$$DS = \frac{(6.66 - NH_2)\times 173}{NH_2 \times 2094}\times 100;$$

$$DS = \frac{MPEGT \times 173}{(100 - MPEGT)\times 2094}\times 100,$$

where: 173 – an average mol. weight of the monosaccharide residue of chitosan (DD 72%);

6.66 – the content of amino groups in chitosan (DD 72%), %.

Both methods gave a very good agreement in DS values and thus confirmed their suitability for analysis of chitosan-*O*-MPEGT graft copolymers.

Spectroscopic and X-ray diffraction measurements

FTIR spectra were recorded with a PERKIN ELMER Spectrum BX spectrometer under dry air at 20 °C by the KBr pellet method. ^{1}H-NMR spectra of the samples dissolved in DMSO were recorded on a TESLA BS 587A 80MHz spectrometer at 29 °C. X-ray diffraction measurements were performed with a Bruker D8 diffractometer equipped with Göbel mirror (primary beam monochromator) for Cu radiation. Continuous scan mode was used in the range of $5° \le 2\,\Theta \le 45°$ with a scan rate of $1°\ 2\,\Theta\ min^{-1}$.

Viscosity measurement

The intrinsic viscosity of the copolymer solutions in aqueous 0.5 M CH_3COOH / 0.5 M CH_3COONa at 25 °C was measured using a dilution-type Ubbelohde viscometer. Reduced viscosity of the solutions at c = 1.25 g/100 ml was measured using a Cannon–Fenske viscometer.

Solubility tests

The solubility of chitosan-O-MPEGT graft copolymers was tested in several organic solvents, distilled water as well as in 0.1 M acetate buffer (pH 4.01), 0.1 M phosphate buffer (pH 7.00) and 0.1 M borate buffer (pH 10.00). The samples were soaked in each solvent at the concentration of 5 mg/ml.

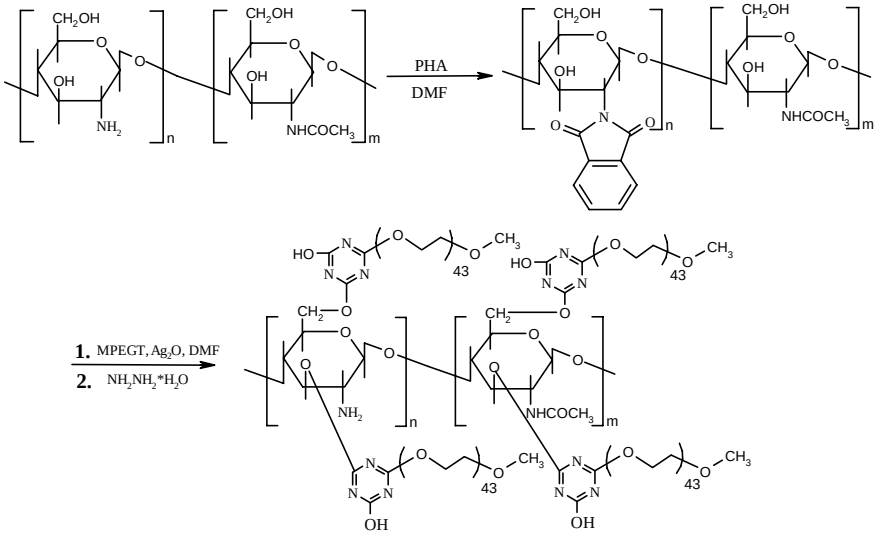
RESULTS AND DISCUSSION

Chitosan contains two hydroxyl groups in every monosaccharide residue and potentially is able to undergo many reactions of alcohols. One of the most common reactions of alcohols is etherification or nucleophilic substitution (S_N2) reaction. It takes place between OH groups of alcohols or between the OH group of an alcohol and the halogen group of an alkyl halide. Alkyl halides (methyl chloride, ethyl chloride, etc.) are the reactants for etherification of cellulose in strongly alkaline aqueous solutions. Such conditions, however, are not acceptable for chitosan for the reasons associated with insolubility of chitosan and, as determined by us, partial deprotection of its amino groups in alkaline solutions.

The present study focuses on etherification of chitosan in polar organic media. The keystone for this pathway is the fact that the N-phthaloyl derivative of chitosan is soluble in several organic solvents (DMSO, DMF, pyridine and dimethylacetamide) [18]. Therefore the N-phthaloyl group is indispensable for both protection of amino functionalities of chitosan and solubilization of the product in an organic solvent, which is of least importance for the further reaction with a halogenated derivative of MPEG.

O-PEGylated chitosan derivatives were synthesized as presented in Scheme. At first, amino groups of chitosan were protected by a 3-fold excess of phthalic anhydride in DMF. It is known [18] that phthalic anhydride excess guarantees a complete N-phthaloylation of chitosan, which was confirmed by IR and ^{1}H-NMR spectra.

"Activation" of MPEG by 2,4,6-trichloro-*s*-triazine was carried out at room temperature in dry benzene by a known method [15] to give 2-*O*-MPEG-4,6-dichloro-*s*-triazine (MPEGT), which was used as a soluble electrophilic scavenger. The use of a non-



Scheme. *O*-PEGylation of chitosan

aqueous solution was essential and allowed to avoid hydrolysis of the product, which is common for 2,4,6-trichloro-s-triazine derivatives in aqueous solutions even at 4 °C [15]. The complete "activation" of MPEG was confirmed by UV-spectroscopy measuring absorbance at λ = 234 nm [19].

MPEGT was attached to *N*-phthaloyl chitosan dissolved in DMF containing Ag_2O dispersion. DMF was ascertained as the best solvent for *O*-PEGylation of chitosan. The other solvents tested were not suitable because of partial oxidation (DMSO) or complexation of MPEGT (pyridine).

Alkaline systems like alkali metal hydroxides, such as sodium hydroxide or potassium hydroxide, or oxides such as silver oxide are a prerequisite for successful etherification of hydroxyl groups of polysaccharides [20]. It was determined by us that the use of alkali metal hydroxides is impossible because of partial deprotection of amino functionalities of chitosan in these conditions. The solubility of silver oxide and the subsequent alkalinity of the solution is considerably lower; that is why silver oxide has proved particularly suitable for the reaction of etherification.

The removal of protective phthaloyl groups was efficiently carried out by treatment with hydrazine monohydrate. This was confirmed by IR spectra of chitosan-*O*-MPEGT graft copolymers that didn't contain absorption at 1770 and 1710 cm^{-1} characteristic of phthalimido groups (Fig.1).

According to chemical analysis data, the obtained chitosan-*O*-MPEGT graft copolymers contained large amounts of PEG units and a predicted amount of free amino groups (Table 1). The degree of *O*-substitution of MPEGT to the monosaccharide residue of chitosan (DS) linearly depends on the molar ratio of MPEGT to chitosan. The reaction between chitosan OH-groups and MPEGT is irreversible and proceeds until consumption of the active functional groups. Any excess of MPEGT is not a prerequisite for the reaction. When chitosan is in excess, chitosan-*O*-MPEGT graft copolymers with different degrees of substitution (8 to 97%) are synthesized (Table). When MPEG is in excess, the degree of *O*-substitution increases up to 200%, *i.e.* both hydroxyl groups in each monosaccharide residue of chitosan are converted into ether groups.

IR spectrum of MPEGT, compared to the spectrum of initial MPEG, exhibits a new absorption band at 1546 cm^{-1}, which is attributed to the triazine ring [21]. IR spectra of chitosan-*O*-MPEGT graft copolymers show the bands characteristic both of chitosan and MPEGT with a much higher absorbance of the groups of the latter (Fig. 1). This isn't unexpected keeping in mind that PEG chains consist of about 44 units. On the other hand, it shows that the degree of substitution of MPEG to chitosan monosaccharide residue is high. Distinct absorption bands at 1110 cm^{-1} (C-O stretching) and 2886 cm^{-1} (C-H stretching) appear and at 3400 cm^{-1} (O-H stretching) decline or almost disappear in the IR spectra of chitosan-*O*-MPEGT copolymers. The intensity of the absorption bands depends on the density of MPEG grafts in the copolymers.

^{1}H-NMR spectra of *N*-phthaloyl chitosan (1), *N*-phthaloyl-*O*-MPEGT chitosan (2) and chitosan-*O*-MPEGT graft copolymers (3) in DMSO-d_6 are given in Fig. 2. Chitosan derivatives containing

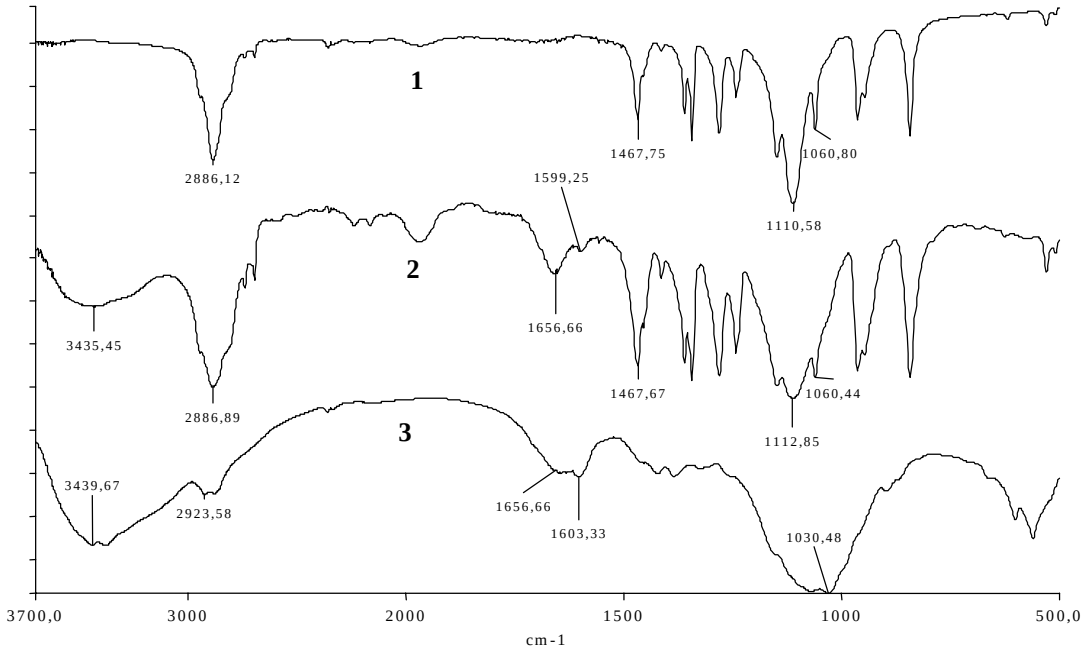


Fig. 1. IR spectra of chitosan-*O*-MPEGT graft copolymers (1 – DS 19%; 2 – DS 23%) and chitosan (3)

Table 1. **Results of the analysis of *O*-PEGylated chitosans**

No	Compound	Amount of		DS, %	$\eta_{sp.}/c^*$, dL/g
		MPEGT, %	NH_2, %		
1	Chitosan	–	6.60	–	–
2	MPEGT	100	–	–	0.082
3	Chitosan-*O*-MPEGT	49.2	3.30	8	0.115
4	Chitosan-*O*-MPEGT	73.29	1.76	23	0.106
5	Chitosan-*O*-MPEGT	84.8	1.03	45	0.095
6	Chitosan-*O*-MPEGT	92.1	0.52	97	0.120
7	Chitosan-*O*-MPEGT	96.3	0.28	192	0.120

*c = 1.25 g/dL.

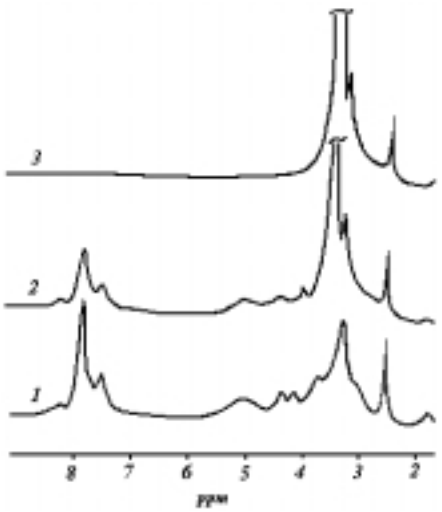


Fig. 2. ^{1}H-NMR spectra of *N*-phthaloyl chitosan (1), *N*-phthaloyl-*O*-MPEGT chitosan with DS 8% (2) and chitosan-*O*-MPEGT graft copolymer with DS 97% (3)

MPEGT grafts are characterized by a new very strong signal at δ = 3.50–3.60 ppm, which is attributed to the oxyethylene groups present in the grafts. The chemical shift at δ = 7.20–7.70 ppm is assigned to the aromatic protons of the phthaloyl group. This signal doesn't disappear in the spectrum of the phthaloylated chitosan-*O*-MPEGT graft copolymer with low DS but becomes weaker (spectrum 2). Chitosan-*O*-MPEGT graft copolymers with high DS (curve 3) contain one dominant signal at 3.50–3.60 ppm, confirming a high density of MPEGT grafts.

X-ray diffraction patterns of chitosan and its graft derivatives are shown in Fig. 3. The original chitosan powder showed two major crystalline peaks at 2θ = 10.8° and 20.0°, which were in good agreement with earlier published data [22]. *N*-phthaloyl chitosan showed only one broad and weak peak at around 2θ = 20°, suggesting that the ability of forming hydrogen bonds was decreased after chemical modification and this chitosan derivative appeared amorphous. The diffraction diagrams of chitosan-*O*-MPEGT graft copolymers didn't depend on DS and were the same as MPEG-2000. The reflection falls at around 2θ = 19.0° and 23.5° were very sharp and narrow, suggesting a high crystallinity of the copolymers. It appeared that chitosan backbones were amorphous but the MPEGT grafts formed a regular structure. No significant differences between the intensities of peaks in X-ray diffraction patterns of chitosan-*O*-MPEGT graft copolymers and MPEG were observed. It appeared that the orientation of crystallites in the both cases was random.

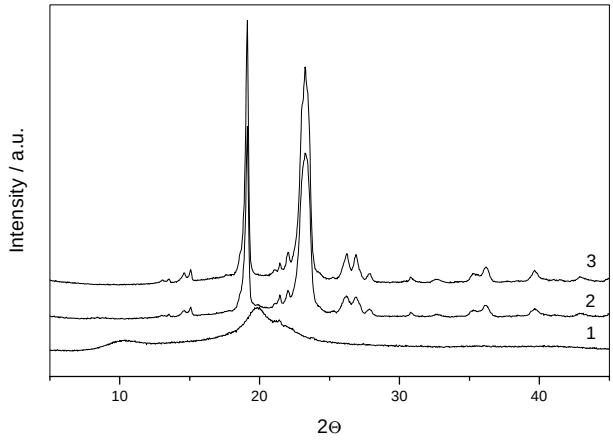


Fig. 3. X-ray diffraction patterns of chitosan (1) and chitosan-*O*-MPEGT graft copolymers with DS 8% (2) and DS 97% (3)

The obtained chitosan-*O*-MPEGT graft copolymers are soluble in water and weakly alkaline aqueous solutions irrespective of copolymer composition. Moreover, these copolymers dissolve immediately like oligomers or low-molecular-weight substances. Chitosan-*O*-MPEGT copolymers become water-soluble irrespective of pH when DS reaches about 15%. The solubility of the graft copolymers in organic solvents is unconventional. Chitosan-*O*-MPEGT copolymers don't dissolve in acetone, ethylacetate, ethanol, chloroform, DMF and toluene at room temperature, but they are easily soluble in the above solvents at elevated temperatures (40–50 °C). After dissolving they don't precipitate under cooling to room temperature. Aliphatic hydrocarbons, carbon tetrachloride and diethyl ether are non-solvents for the grafts copolymers.

Reduced viscosity $\eta_{sp.}/c$ of 1.25% aqueous solutions of the copolymers in 0.5M CH_3COOH/0.5M CH_3COONa at 25 °C is very low and similar to that of MPEGT (Table 1). It means that grafting of

MPEGT onto chitosan drastically decreases solution viscosity of the latter. For comparison, intrinsic viscosity [η] of chitosan (M_r 400000, DD 72%) in the same acetate buffer is 8.4 dL/g. The reduced viscosity depends on copolymer composition and reaches its minimal value at moderate degree of substitution. The reduced viscosity of the solutions of PEGylated chitosans is slightly increasing under dilution of the solutions. This abnormal behaviour is typical for highly branched copolymers. Brush structure of the copolymers is responsible for very high average coil density of macromolecules.

Low viscosity of chitosan-*O*-MPEGT graft copolymers could be related to hydrogen bonding and aggregation phenomena. Poor solubility of chitosan and high viscosity of its solutions is explained by partially crystalline structure of this polymer and very tight hydrogen bonding between amino and hydroxyl groups [2]. Grafting of PEG chains separates chitosan backbones, collapses order characteristic to chitosan solutions and drastically decreases hydrogen bonding. It is known also [23] that macromolecules of chitosan – PEG copolymers may form aggregates in an aqueous solutions. Compacticity of these aggregates depends on density of PEG chains and possibly reaches maximal value at moderate DS. Just these copolymers have lowest value of reduced viscosity (Table 1).

Chitosan-*O*-MPEGT graft copolymers containing free amino groups may be applied in biotechnology or in biomedical systems. The modified chitosans are expected to be recognized by polysaccharides and microorganisms because of unchanged fundamental skeleton of these poly(glucose amines). On the other hand, due to water solubility and free amino groups, *O*-PEGylated chitosans can act as useful intermediates for further chemical modifications.

CONCLUSIONS

1. Novel chitosan graft copolymers with various density of poly(ethylene glycol) brushes were synthesized using chitosan and poly(ethylene glycol) 2000 monomethyl ether (MPEG). MPEG was “activated” by 2,4,6-trichloro-*s*-triazine resulting in very convenient and active intermediate.

2. Chitosan-*O*-MPEGT graft copolymers were suggested having crystalline structure similar to that of MPEG. *O*-PEGylated chitosans were soluble in water and weakly alkaline aqueous solutions. Intrinsic viscosity of aqueous solutions of the graft copolymers was extremely low and similar to that of MPEG 2000.

Received 08 December 2003
Accepted 29 December 2003

References

1. G. Moore and G. Roberts, *Int. J. Biol. Macromol.*, **4**, 246 (1982).
2. S. Nishimura et al., *Macromolecules*, **24**, 4745 (1991).
3. J. M. Harris (Ed.). Poly(ethylene glycol) Chemistry: Biotechnical and Biomedical Applications. New York: Plenum Press, 1992.
4. M. Sugimoto et al. *Carbohydrate Polymers*, **36**, 49 (1998).
5. H. Saito et al. *Macromol. Rapid Commun*, **18**, 547 (1997).
6. K. Kurita et al. *Polymer Bulletin*, **42**, 387 (1999).
7. M. D. Bentley, M. J. Roberts and J. M. Harris, *J. Pharm. Sci*., 87, 1446 (1998).
8. D. Siksniene et al., *Biochem. Biotechn. Electronic Express*, **2**, 23 (1993).
9. M. Yamasaki et al, *Biotechnology Techniques*, **12**, 751 (1998).
10. J. Boada et al. *Colloids and Surfaces*, **182**, 191 (2001).
11. B. Martel et al. *J. Polym. Sci. Part A*, **39**, 169 (2001).
12. M. Marzona, G. Modica, *Textil. veredlung*, **12**, 806 (1971).
13. E. Levin and N. Vinogradova, *Zh. Prikl. Spektr*., **4**, 330 (1966) (in Russsian).
14. A. Petrov and A. Remizov, *Zh. Org. Chimii*, **40**, 1611 (1969) (in Russian).
15. A. Abuchowski et al., *J. Biol. Chem*., **252,** 3578 (1977).
16. J. G. Domszy and G. A. F. Roberts, *Makromol Chem*., **186**, 1671 (1985).
17. A. Nag, G. Mitra and P. C. Ghosh, *Anal. Biochem*., **237**, 224 (1996).
18. K. Kurita, *Progr. Polym. Sci*, **26**, 1921 (2001).
19. J. Boada, M. Callardo and J. Estelrich, *Anal. Biochem*., **253**, 33 (1997).
20. US Pat. No. 4803263 (1989).
21. Y. Yu, Y. Zhuang and Q. Zou, *Chemosphere*, **44**, 1287 (2001).
22. S. Nishimura et al., *Carbohydrate Research*, **306**, 427 (1998).
23. T. Ouchi, H. Nishizawa and Y. Ohya, *Polymer*, **39**, 5171 (1998).

N. Gorochovceva, R. Kulbokaitë, R. Juðkënas, R. Makuðka

CHITOZANO IR POLIETILENGLIKOLIO SKIEPYTØJØ KOPOLIMERØ, TURINÈIØ TRIAZINO ÞIEDÀ, SINTEZË IR TYRIMAS

S a n t r a u k a

Susintetinti nauji chitozano-*O*-polietilenglikolio skiepytieji kopolimerai, turintys skirtingà skiepø tankà. Prieð kovalentiðkai prijungiant prie chitozano, polietilenglikolio monometileteris (MPEG) buvo „aktyvuojamas“ trichlortriazinu. Chitozano eterifikacijos reakcija buvo vykdoma sidabro oksido suspensijoje dimetilformamide. Chitozano-*O*-polietilenglikolio skiepytieji kopolimerai buvo iðtirti, naudojant FTIR ir MBR spektroskopijà, rentgeno spinduliø difrakcijà ir elementinæ analizæ. Skiepytieji chitozano kopolimerai tirpûs vandenyje plaèiame pH verèiø intervale. Ðiø kopolimerø vandeniniø tirpalø klampos skaièius yra labai maþas ir artimas MPEG klampai.